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Characterization and Inhibitor Studies of Chitinases from a Parasitic Blowfly (*Lucilia cuprina*), a Tick (*Boophilus microplus*), an Intestinal Nematode (*Haemonchus contortus*) and a Bean (*Phaseolus vulgaris*)†

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Abstract: The molecular weight pattern and the stage-specific activities of chitinases from the blowfly *Lucilia cuprina*, the tick *Boophilus microplus* and the intestinal nematode *Haemonchus contortus* were examined. Chitinolytic enzymes could be detected in all parasite species tested, but the activity was different between the stages. Highest chitinolytic titers were found in blowfly pupae (83 kDa, 118 kDa), hatching larvae of ticks (58 kDa, 94 kDa) and nematode eggs (43 kDa). Leaves from ethylene-treated bean plants *Phaseolus vulgaris* expressed two basic Class I chitinases (Ia, Ib) of 34 kDa, differing in their amino acid sequences at residue 33 and 34 (Ia: glycine, proline; Ib: lysine, aspartic acid). Inhibitor studies with blowfly pupae revealed that allosamidin (IC_{50} = 0·32 (±0·02) μM) was by far the best inhibitor when compared with various amino sugar derivatives. This compound also inhibited chitinases from tick larvae (IC_{50} = 0·69(±0·10) μM) and nematode eggs (IC_{50} = 0·048(±0·0045) μM) specifically. Whereas Class Ia chitinase from bean leaves was inhibited only up to 18% by 10 μM allosamidin, it had an IC_{50} of 1(±0·14) μM for the Ib type, which is the first plant chitinase described to be highly sensitive to allosamidin.

Key words: allosamidin, *Boophilus microplus*, chitinase, *Haemonchus contortus*, inhibitor, *Lucilia cuprina*, parasites, *Phaseolus vulgaris*

1 INTRODUCTION

From a theoretical point of view chitin synthesis and degradation seem to be highly specific targets for insecticides,[1,2] since the associated processes are essential for arthropod pests, but do not occur in vertebrates. In fact, the inhibition of chitin synthesis by benzoylphenylureas is a successful example of this strategy.[1,3,4] Whereas inhibition of chitin synthesis is an established means of insect pest control, this does not hold true for chitinolysis, Until very recently this was due to the fact that no selective inhibitors were available. By the discovery of allosamidin, a specific chitinase inhibitor isolated from *Streptomyces* sp. by Sakuda *et al.* in 1986,[5] the prospects of exploiting this target for the development of new parasiticides have become more attractive. In this context the discovery of chitinases or chitinase-like enzymes in protozoa as transmission factors[6] and possible nematode vaccination antigens[7,8] even extends this approach to new taxa. Chitinases are also widely distributed among various species of higher plants.[9] In response to wounding, abiotic stresses, and pathogen

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attack, the enzymatic activity of chitinases is induced in different plant tissues. So far there has been no direct evidence for the role of chitinases within the network of plant defence reactions, but data obtained with transgenic plants constitutively expressing a chitinase gene showed an enhanced resistance to fungal pathogens.[10] Broadly active inhibitors of chitinases might not only inhibit parasite enzymes but could also affect the role of chitinases in the plant defence mechanism. In this context, of particular interest is information about the similarity of plant and insect chitinases with regard to their substrate and inhibitor specificity in order to assess any possible potential interference of such pesticides with natural plant defence reactions.

2 EXPERIMENTAL METHODS

2.1 Rearing of insects

Lucilia cuprina Wied. at different developmental stages were selected from synchronized cultures (±5 h). For testing of four-day-old pupae, 50 fly larvae were sorted at the prepupal stage (120 h) and kept in plastic dishes for a further 96 h at 20–22°C and 75% relative humidity (RH). Insects were staged according to Filshi.[11] About 400 eggs from fully engorged female one-host *Boophilus microplus* Can. ticks (pyrethroid-resistant Parkhurst strain) were collected from cattle and kept at 26°C in the dark at 75% RH. Eggs from days 10 and 17, as well as hatching larvae, engorged larvae and fully sucked adults were used in the investigations. About 2000 eggs from the nematode *Haemonchus contortus* Rud. were purified from faeces according to Roberts and O'Sullivan[12] and hatched for L3 larvae. Adults of both sexes were collected from freshly killed sheep abomasum and used directly in the chitinolytic assays.

2.2 Plant growth conditions

Seeds of *Phaseolus vulgaris* L. cv. Saxa were purchased from Samen Nebelung, Heidelberg. Bean plants were grown in controlled-environment chambers for three weeks. Ethylene treatment was performed for two days by spraying with a solution of ethephon (2-chlorethylphosphonic acid, Sigma; 1 mg ml^{-1}) and enclosing the plants in plastic bags.

2.3 Sample preparation

In general, samples from parasites were prepared by homogenization in sodium phosphate buffer (0·1 M, pH 7·0, 100 g $litre^{-1}$ glycerol) with an all-glass homogenizer (*L. cuprina*) or in a liquid-nitrogen-cooled mortar (*B. microplus*, *H. contortus*). All homogenates were subsequently ultrasonicated (3 × 5 s, 50 W) and centrifuged (1 h; $10^5 g$; 4°C). Aliquots of supernatants were diluted to appropriate protein concentrations and either stored at −25°C or used directly for enzyme assays. Enzyme activity in stored extracts was stable for at least six months. Column chromatography was carried out on Beckman Ultra Spherogel™-SEC 3000 in sodium phosphate buffer (0·1 M, pH 7·0) at 0·5 ml min^{-1} flow rate on a Waters HPLC system. Fractions (100 µl) were collected and tested as described in Section 2.4. Relative molecular weights were calculated from linear regression curves from standard proteins. Plant chitinases were purified from ethylene-treated bean leaves. Frozen plant material (200 g) was homogenized in sodium citrate buffer (0·1 M, pH 5·0, EDTA 1 mM, DTT 10 mM; 400 ml). The homogenate was centrifuged (15 × $10^3 g$; 10 min) and the sediment was re-extracted with sodium citrate buffer (400 ml). Supernatants were combined, filtered and ammonium sulfate was added to give 80% saturation. The resulting protein precipitate was redissolved in sodium acetate buffer (20 mM, pH 4·8, EDTA 1 mM, DTT 1 mM; 200 ml), clarified by centrifugation and passed through a Sephadex G-100 column (6 × 22 cm) equilibrated with the same buffer. Protein-containing fractions were pooled, dialyzed against sodium acetate buffer pH 4·8 and applied to an *S*-Sepharose Fast Flow column (Pharmacia, 3 × 13 cm). Unbound protein was eluted with 20 mM sodium acetate pH 4·8, followed by a linear gradient from 0 to 0·5 M sodium chloride. In a final purification step, enzyme activity-containing fractions were subjected to a second cation-exchange chromatography step, using a Mono S HR 10/10 column (Pharmacia). The column was equilibrated with sodium acetate (20 mM, pH 5·0, EDTA 1 mM, DTT 1 mM) and bound proteins were eluted with a linear gradient (150 ml) of increasing sodium chloride concentration (0 to 50 mM). Chitinase activity eluted in two separated peaks. Peak fractions were pooled, dialyzed against 50 mM ammonium carbonate, lyophilized and kept for further investigations. Determination of molecular weights and homogeneity was performed by sodium dodecylsulfate gel electrophoresis.

2.4 Enzyme assays

N-Acetyl-β-D-glucosaminidase activity was determined by measuring the liberation of *p*-nitrophenol (pNP) from *p*-nitrophenyl *N*-acetyl-β-D-glucosaminide. Citrate phosphate-buffer (0·2 M; pH 5·5; 50 µl), with the appropriate substrate concentration, and homogenate in the same buffer (50 µl) were mixed and incubated at 25°C for 10 min, then the reaction was stopped by addition of sodium hydroxide (0·01 M; 700 µl). The absorbance of the phenolate anion was measured at 410 nm and expressed as mM *p*-nitrophenol mg^{-1} protein min^{-1}. If necessary, exochitinases were inhibited by PUGNAG

TABLE 1
Stage Specificity of Endochitinolytic Activity and Molecular Weight Pattern of the Fly *Lucilia cuprina*, the Cattle Tick *Boophilus microplus*, the Sheep Nematode *Haemonchus contortus* and the Plant *Phaseolus vulgaris*

Species	*Stage*	*Time of development (days)*	*Endochitinolytic activity*[a,b] *(%)*	*Apparent molecular weight of chitinolytic enzymes (kDa)*
L. cuprina	Eggs	0·1	100	96; 75
	Larvae 1st instar	0·3	201	n.d.[d]
	Larvae 2nd instar	0·9	207	96; 54
	Larvae 3rd instar	2·1	85	n.d.
	Pupae	3·2	117	n.d.
		5	240	118; 83; 54; 38; (26)
	Adults	8	113	75; 30
B. microplus		6	100	n.d.
	Eggs	10	875	114
		17	18 150	60; 30
	Hatching larvae	27	251 500	94; 58; 36
	Larvae	29	320	97; 62; 38
	Adults	49	463	78
H. contortus	Eggs	0·1	100	43; (56)
	Larvae	1	19	43
	Adults	42	6	not detectable
P. vulgaris[c]	Leaves	21	100	34

[a] Endochitinolytic activity was determined by sample incubation with 2-acetamido-2-deoxy-D-glucono-1,5-lactone (100 μM) to inhibit *N*-acetyl-β-D-glucosaminidases (exochitinases).
[b] 100% value corresponds to the following specific enzyme activities: (pmol MUF min^{-1} mg^{-1} protein): *L. cuprina:* 173; *B. microplus:* 0·2; *H. contortus:* 221; *P. vulgaris* (pure enzyme): 2700.
[c] Purification procedure of endochitinolytic activity of *P. vulgaris* was followed using a determination with tritium-labelled chitin.
[d] n.d. = not determined.

(*O*-(2-acetamino-2-deoxy-D-glucopyranosyliden)amino-*N*-phenylcarbazone). Chitinolytic activity after column chromatography of various parasite stages was determined with a highly sensitive fluorescence assay slightly modified from that of Gooday *et al.*[13] Diluted extract (50 μl) and MUF-chitotriose (4-methylumbelliferyl *N,N',N''*-triacetyl-β-chitotrioside; Calbiochem; 0·08 M, 50 μl) were incubated for 10–15 min at 20°C. Fluorescence of released MUF was measured in a Kontron spectral fluorometer at 350 nm excitation and 440 nm emission wave length after enhancement of fluorescence at pH 11 (100 mM sodium hydroxide + glycine). For comparison, incubation conditions were maintained constant within species. Inhibitors for endo- and exochitinase were added dissolved in dimethylsulfoxide (5 μl); control experiments revealed no effect of the solvent on the enzymatic activities.

2.5 Protein determination and amino acid analysis

Protein content was determined according to Bradford[14] using bovine serum albumin as standard. Determination of NH_2-terminal amino acid sequences by Edman degradation was carried out on an Applied Biosystems 476A sequencer.[15] Phenylthiohydantoin amino acid derivatives were identified on-line by HPLC.

2.6 Statistical analysis

K_m values were computed according to Lineweaver-Burk.[16] For determination of IC_{50} values a logit–log transformation was performed. The data were fitted to straight lines by the linear least squares method and IC_{50} values were calculated using Sigma Plot (Jandel Scientific).

2.7 Inhibitors

The synthesis of other inhibitors (see Table 2) has been, or will be, published as follows: compound **II**;[17,18] compounds **III** and **IV**;[19,20] compounds **V**, **VIII**;[21] compound **VI**.[22] Compound **VII** was prepared by the

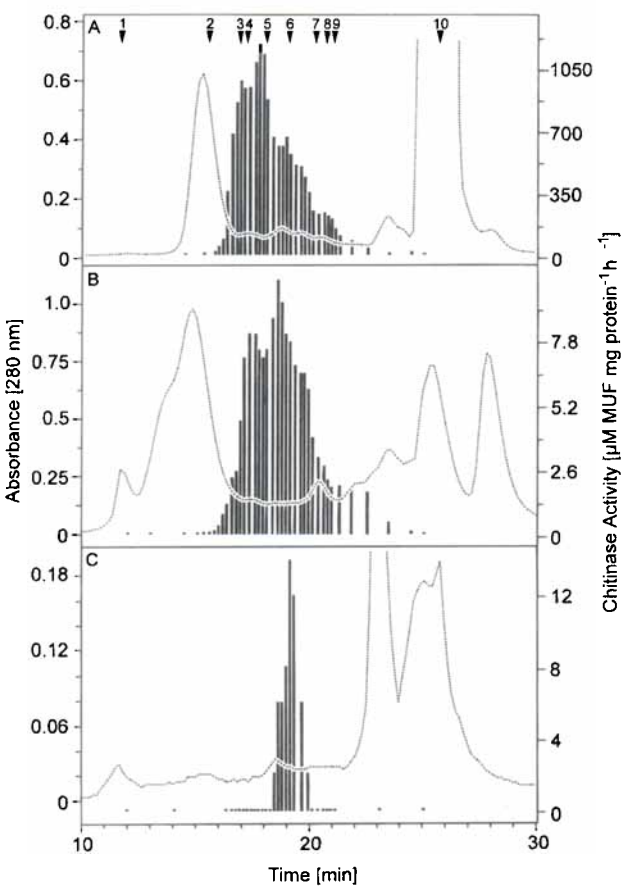


Fig. 1. Molecular sieve separation of chitinases from (A) five-day-old *Lucilia cuprina* pupae, (B) newly hatched (day 27) *Boophilus microplus* larvae and (C) *Haemonchus contortus* eggs. In Figs 1(A) to 1(C), chromatographic separation was performed of cytosolic extracts of parasites. Molecular weight standards (▼) were 1: void volume, 2: ferritin, 3: aldolase, 4: phosphorylase B, 5: bovine serum albumin, 6: ovalbumin, 7: carboanhydrase, 8: chymotrypsinogen A, 9: cytochrome C, 10: inclusion volume. Solid bars represent enzyme activity, dotted lines represent absorbance at 280 nm.

method of Matta and Bahl.[23] Chitotriose and chitotetraose were either prepared by acid-catalyzed acetolysis of chitin[24] followed by chromatography and *O*-deacetylation, or purchased from Calbiochem.

3 RESULTS

The activity of chitinolytic enzymes was examined during the developmental stages of parasites to determine the time period with the highest titer and the pattern of differential expression of enzymatic activity. Chitinolytic enzymes could be demonstrated in all species tested. As presented in Table 1, the activity is different among the tested parasite stages. Specific activity in *L. cuprina* was highest in pupae shortly before hatching. High activity was also observed in 1st- and 2nd-instar larvae, declining in the 3rd instar and then constantly increasing up to maximum value in pupae. The enzymes were separated on the molecular sieves column to yield the stage-specific pattern of activity (Fig. 1, Table 1). Cytosolic extracts from eggs showed main chitinolytic activity in the 75 kDa and 96 kDa fractions. The chromatograms from extracts displayed high activity at 96 kDa and 54 kDa for larvae and at 83 kDa and 118 kDa for pupae with some minor additional activity in later-eluting fractions (Fig. 1(A)). In extracts from adults, chitinolytic activity was demonstrated predominantly at 30 kDa and 75 kDa. Chitinolytic activity in *B. microplus* increased drastically from newly deposited eggs to hatching larvae. Enzyme activity in older larvae, nymphs and in adults was comparably low. In adults, about 50% of the total chitinolytic activity appears to be associated with exochitinases (inhibition by 100 µM PUGNAG). Several chitinolytic enzymes of ticks were separated by HPLC on molecular sieves. Enzyme activity in 10-day-old eggs was detected

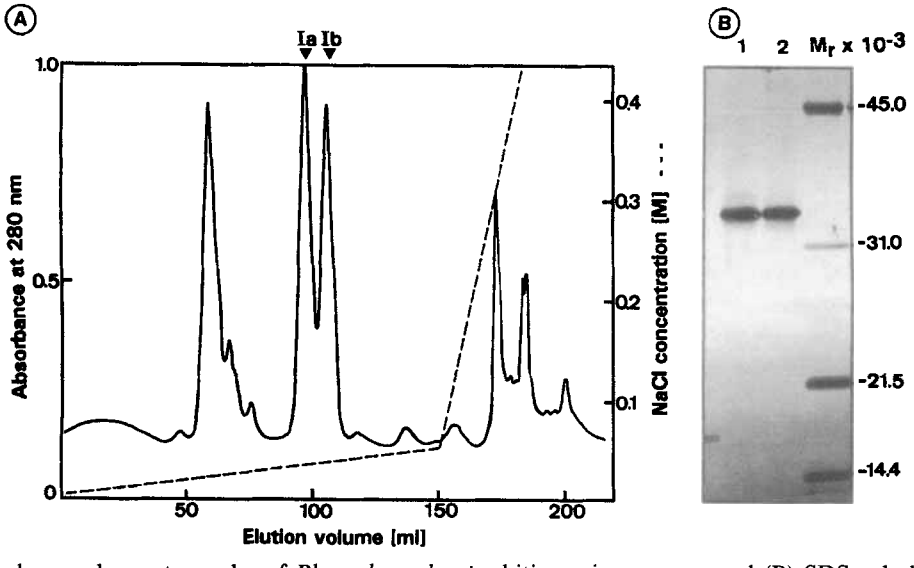


Fig. 2. (A) Cation exchange chromatography of *Phaseolus vulgaris* chitinase isoenzymes and (B) SDS gel electrophoresis of isoenzyme fractions. In Fig. 2(A) chitinase-containing fractions are indicated by arrows (Ia and Ib). In Fig. 2(B) electrophoretic separations of fraction Ia (lane 1), Ib (lane 2) and marker proteins ($M_r \times 10^3$) are shown.

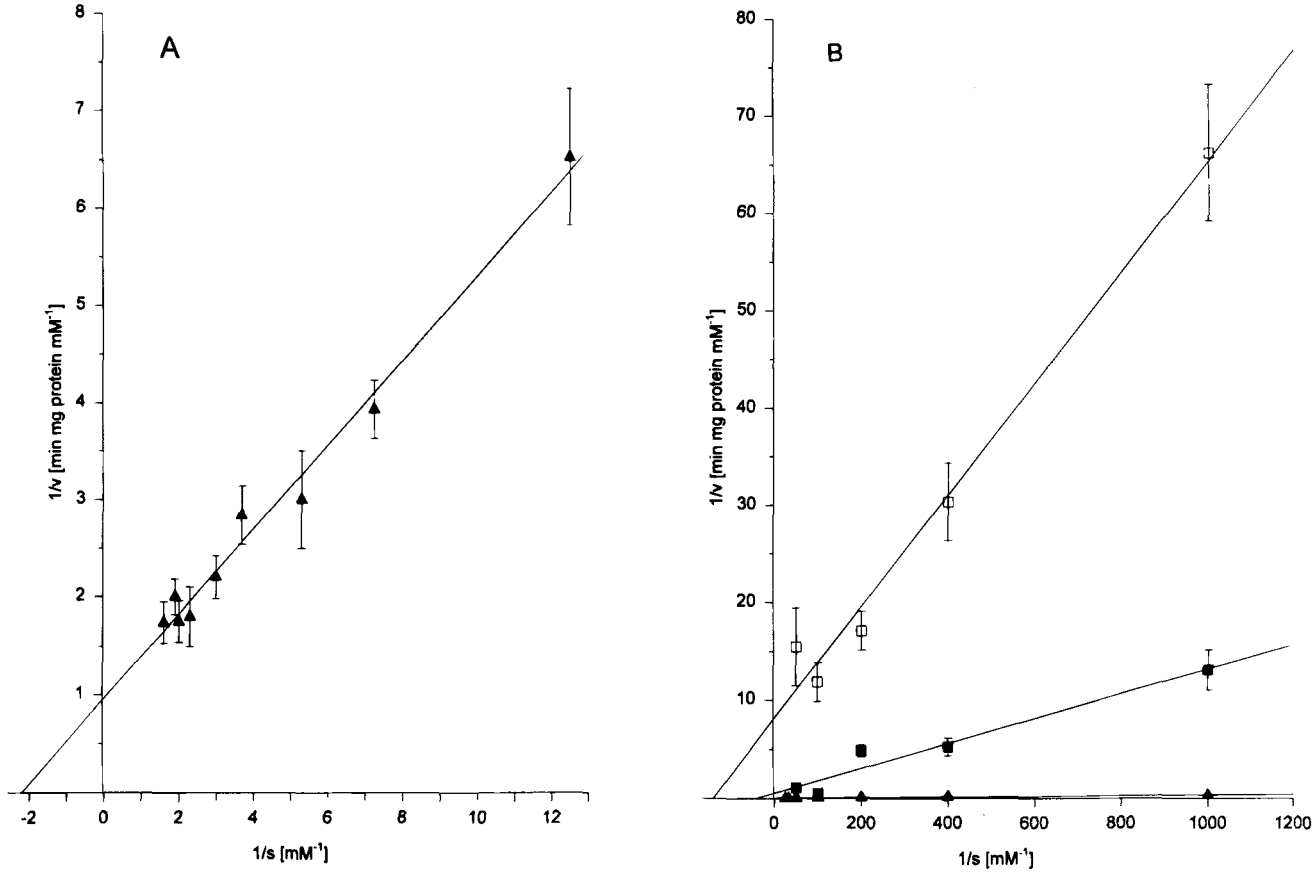


Fig. 3. Lineweaver–Burk plots of kinetic properties of (A, ▲) *Lucilia cuprina* *N*-acetyl-β-D-glucosaminidase and (B) chitinases from (▲) *Lucilia cuprina*, (■) *Phaseolus vulgaris* Ib and (□) Ia. The values are means of five determinations ± S.D.

predominantly at 114 kDa. The main activity peaks shifted to about 30 kDa and 60 kDa in 17-day-old eggs and activities at 36 kDa, 58 kDa and 94 kDa were observed in newly hatched larvae (Fig. 1(B)). Similar molecular weights with a more pronounced peak at 45 kDa were found for chitinolytic enzymes in the nymphs of the tick *Rhipicephalus sanguineus* Latr. (data not shown). Chitinolytic enzymes from adult tick cytosolic extracts showed a molecular weight essentially at 78 kDa. In contrast to earlier observations[25] from studies using colloidal chitin as substrate, all stages of the nematode *H. contortus* displayed chitinolytic activity. In adults, total activity was too low for further characterization following HPLC separation. Chitinase activity was detected at 43 kDa in larvae and eggs (Fig. 1(C)).

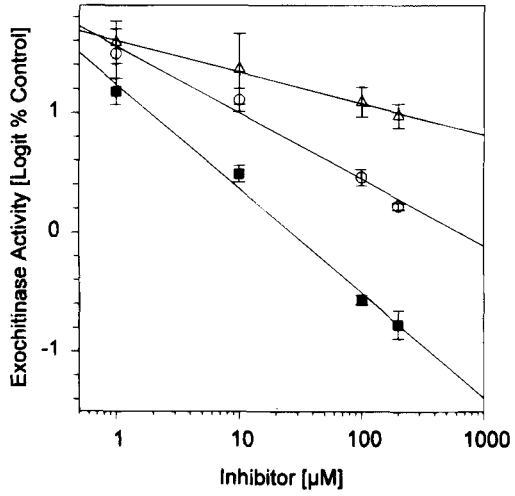
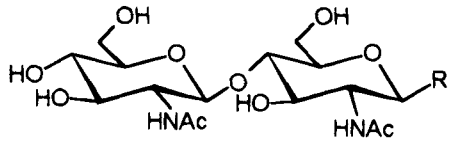
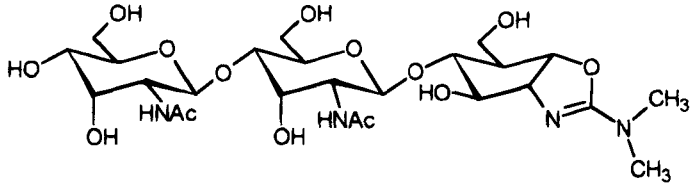
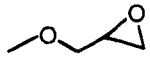
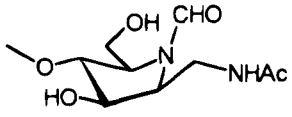
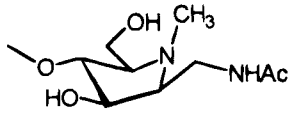
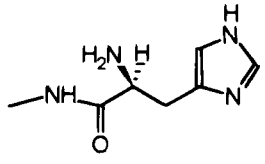
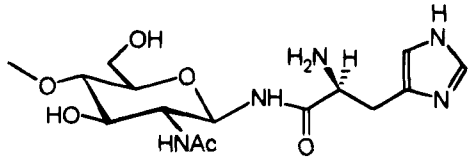
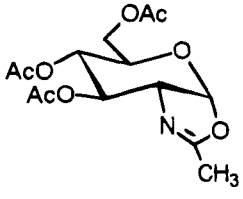
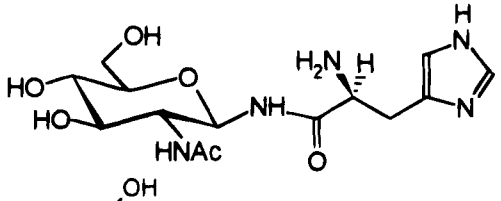
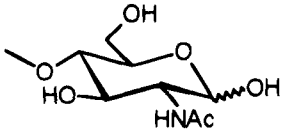
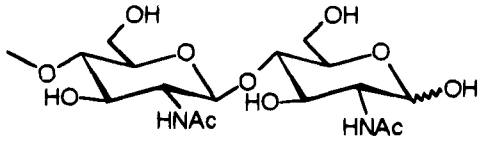


Fig. 4. Influence of (△) *N,N'*-diacetylchitobiose, (■) 2-acetamido-2-deoxy-D-glucono-1,5-lactone and (○) methyl 2-(2',4'-dinitrophenyl)-amino-2-deoxy-α-D-glucopyranoside on *N*-acetyl-β-D-glucosaminidase from *Lucilia cuprina* pupae. The values are means of three determinations ± SEM. Data are presented as percentage activity of control after logit–log transformation. Control values corresponded to 0·62 mM pNP mg^{-1} protein min^{-1}.

Plant chitinases were isolated from ethylene-treated bean leaves. Thereby, two chitinase activity-containing fractions were separated by high performance cation exchange chromatography (Fig. 2(A)). As judged by SDS-PAGE, both peak fractions (termed chitinase Ia and Ib) contained a single polypeptide with an apparent molecular weight of 34 kDa (Table 1). Determination of the NH_2-terminal amino acid sequence confirmed the enzymes as basic class I chitinases (Fig. 2(B)).[26,27] The amino acid sequences of both isoenzymes were identical up to residue 32, and corresponded to the sequence previously described for the mature ethylene-induced chitinase protein.[28] By partial amino-terminal sequencing of the purified proteins two amino acid substitutions were discovered: Ia:

TABLE 2
Inhibitory Effect of Different Compounds on Chitinase Activity of *Lucilia cuprina* pupae

Compound		*Half-maximal*[b] *inhibition* (*μM*) (±*S.D.*) (*n* = 3)	*Inhibition at 1 mM*[c] (%) (±*S.D.*) (*n* = 3)
			
I (allosamidin)		0·32 (±0·02)	100
II	R = 	290 (±62)	92 (±4)
III	R = 	260 (±52)	79 (±14)
IV	R = 	940 (±113)	51 (±17)
V	R = 	>1000	38 (±11)
VI	R = 	240 (±29)	71 (±22)
VII		>1000	37 (±12)
VIII		>1000	6 (±4)
Chitotriose	R = 	200 (±38) apparent	81 (±32) apparent
Chitotetraose	R = 	280 (±41) apparent	89 (±22) apparent

[a] General structure.
[b] Inhibition data were calculated after logit-log transformation.
[c] Control value corresponds to 4·46 nmol h^{-1} mg^{-1} protein.

TABLE 3
Comparison of Kinetic Properties and Inhibitor Sensitivity of Chitinases from *Phaseolus vulgaris* and *Lucilia cuprina*

Source	Chitinase type	K_m μM (±S.D.)[a]	V_{max} (mM mg^{-1} protein min^{-1})	Inhibition (%)[b] Chitotriose (4 mM)	Inhibition (%)[b] Allosamidin (10 μM)
P. vulgaris	Ia	7 (± 1·8)	0·12	11	18
P. vulgaris	Ib	24 (±1·3)	1·88	61	71
L. cuprina	from pupae	43 (±7)	133·23	100	99

[a] $n = 3$.
[b] Control values corresponded to 80 and 242 pmol h^{-1} μg^{-1} protein for *P. vulgaris* type Ia and Ib, and to 3456 pmol h^{-1} mg^{-1} protein for *L. cuprina*.

EQCGRQAGGA LCPGGNCCSQ FGWCGSTTDY CG***PG***CQSQCGG and Ib: EQCGRQAGGA LCPGGNCCSQ FGWCGSTTDY CG***KD***CQSQCGG. At residues 33 and 34 the glycine and proline residues in the chitinase Ia isoenzyme were exchanged for lysine and aspartic acid in the sequence of chitinase Ib.[29,30]

In order to establish an easily accessible primary screening system for the investigation of chitin degradation inhibitors, *L. cuprina* pupal chitinolytic enzymes were further characterized as shown in Fig. 3. The endochitinase activity exhibits a K_m value of 43(±7) μM (Fig. 3(B)). Determination of isoelectric points by direct incubation with fluorogenic substrate after IEF-gel electrophoresis revealed acidic pI values of 4·8, 5·9, 6·1 and 6·5

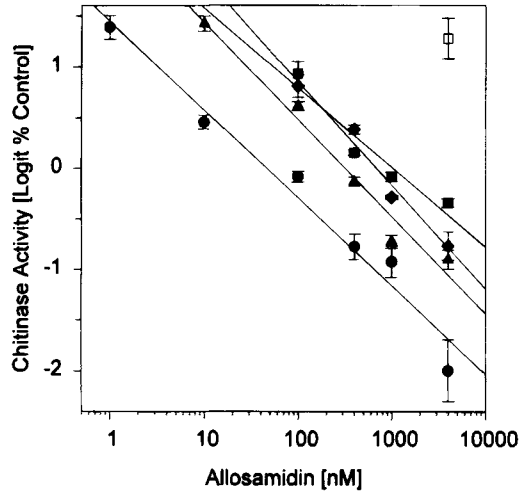


Fig. 5. Inhibition by allosamidin of chitinases from (●) *Haemonchus contortus* eggs, (▲) *Lucilia cuprina* pupae, (◆) *Boophilus microplus* larvae and (□) *Phaseolus vulgaris* type Ia and (■) Ib. The values are means of three determinations ± SEM. Data were presented as percentage activity of control after logit–log transformation. Control values corresponded to 380 pmol h^{-1} mg^{-1} protein for *H. contortus* cytosol, 3·5 nmol h^{-1} mg^{-1} protein for *L. cuprina* cytosol, 121 pmol h^{-1} mg^{-1} protein for *B. microplus* cytosol and 80 pmol h^{-1} mg^{-1} protein for type Ia as well as 242 pmol h^{-1} mg^{-1} protein for type Ib from *P. vulgaris* purified enzyme.

(data not shown) indicating chitinase isoenzymes in the pupae, as has also been demonstrated in other insects.[31] The analysis of *N*-acetyl-β-D-glucosaminidase revealed a K_m value of 311(±22) μM (Fig. 3(A)) and was highly sensitive to 2-acetamido-2-deoxy-D-glucono-1,5-lactone, with an IC_{50} value of 26(±4) μM (Fig. 4) whereas chitinase was not effected at 250 μM by this inhibitor. Chitobiose and methyl 2-(2′,4′-dinitrophenyl)amino-2-deoxy-α-D-glucopyranoside[32] significantly inhibited exochitinolytic activity only at high concentrations with an IC_{50} value of 764(±47) μM (Fig. 4). Chitobiose showed no significant inhibition of *N*-acetyl-β-D-glucosaminidase activity.

In contrast to exoglycosidases in general, only few data are available on inhibitors specific for enzymes cleaving glycosidic bonds in polysaccharides, in particular chitin. In order to gain more detailed information on the substrate specificity of endochitinases, various inhibitors containing chitobiose or chitotriose units in combination with an aglycon terminal unit were synthesized.[17–24] Their formulae and activities are shown in Table 2. Allosamidin (**I**) was found to be by far the best inhibitor when compared with other derivatives. From the compounds tested, **II**, **III** and **VI** revealed an inhibitory activity which was in the range of the apparent IC_{50} of chitotetraose, with compound **II** expressing a nearly full inhibitory effect at 1 mM. For the monosaccharides (**VII**, **VIII**) and the chitobiosyl histidine amide (**V**), only weak effects were observed at 1 mM. Based on these results further investigations were undertaken to determine the effect of allosamidin on parasite and, in particular, plant chitinases. The K_m values of the purified plant chitinase (Fig. 3(B)), as well as the effects of chitotriose and allosamidin, were determined with soluble fluorogenic substrate and are shown in Table 3.

Whereas the plant chitinase Ia with a K_m value of 7(±1·8) μM was nearly insensitive to inhibition with allosamidin and chitotriose, for the type Ib isoenzyme, with a K_m value of 24(±1·3) μM, an IC_{50} value of

1(±0·14) μM (Fig. 5) was determined. Enzymes from *L. cuprina* pupae were inhibited with an IC_{50} value of 320(±22) nM. Allosamidin also displayed a pronounced inhibitory effect on chitinases from ticks with a half-maximal inhibition at 0·69(±0·1) μM. The nematode enzymes were found to be the most susceptible with an IC_{50} value of 0·048(±0·0045) μM (Fig. 5).

4 DISCUSSION

Profound changes in the titer of chitin-degrading enzymes during premoult, before exuviation and during pupal development are a common feature in many arthropods and have also been described for several other species.[33,34] An increase in chitinolytic enzymes during premoult or hatching, as obtained for *L. cuprina*, *B. microplus* and *H. contortus*, is necessary for the partial degradation of the old cuticle or egg shell and have been observed also in a variety of arthropods[34,35] and infective *Ascaris suum* Goeze eggs, as well as hatching fluid.[36] Molecular weights from arthropod chitinases ranged from 9 kDa to 119 kDa[37] with most chitinases in the 40–50 kDa area, e.g. *Drosophila hydei* Sturt.[38] and *Manduca sexta* L.[39] The discovery of chitinases in *Onchocerca gibsoni* Cle. & John.,[13] *H. contortus* (43 kDa and 56 kDa, Table 1) or chitinase-like antigens in microfilariae from *Brugia malayi* Brug[7] (70 kDa and 75 kDa) and larval extracts from *Wucheria bancrofti* Cob.[8] (43 kDa) indicates that chitin may play a role not only in nematode egg development but also during other stages of nematode life cycle. In this context, the extremely complex spectrum of molecular sizes, isoenzyme characteristics, stage- and interspecific species differences might not only reflect the high variation of organisms specifically regulating chitin degradation but is also dependent on the various assay conditions used.[40]

Plant chitinases, which are widely distributed among higher plants, are a structurally diverse group of enzymes differing in their physical properties and locations in plant compartments. Chitinase genes are expressed either constitutively or after induction by organic molecules, salts, wounding or pathogen infection. So far the physiological function of chitinases is unknown, although specific isoforms are considered to play a role in pollination and embryo development.[41,42] As described, chitinase activity in leaves of bean plants increases 30-fold after treatment with exogenous ethylene.[28] It has been reported that bean chitinases are encoded by a multigene family consisting of approximately four members, and that two different basic isoenzymes are induced by ethylene.[29,30] We isolated two basic chitinases from ethylene-induced bean leaves by cation exchange chromatography. The identities of these two isoenzymes have been determined by amino-terminal sequence analysis of 40 residues. The amino acids at position 33 and 34 indicate that the proteins are derived from two different chitinase mRNAs, CH5B and pCh 18, respectively.[29,30] In order to simulate natural stress responses important during pathogen attack, type Ia and Ib (Table 3) chitinases of *P. vulgaris* were induced by ethylene treatment. So far no inhibitory effects of allosamidin against constitutively expressed plant chitinases have been demonstrated.[1,43] The large difference in susceptibility of chitinases Ia and Ib (Table 3) of *P. vulgaris* indicates that, even within one organism, chitinase isoenzymes are expressed after stress induction with different catalytic properties and inhibitor susceptibility resulting from a few amino acid substitutions at positions relevant for the catalytic activity. These results suggest that types of chitinases can be induced in plants which resemble insect chitinases to some extent. Therefore these enzymes have to be investigated carefully after application of 'antichitinase' pesticides in order to prevent interference with their assumed function during defence reactions.

In order to evaluate the suitability of substances interfering with chitin degradation, various types of hexosaminidase inhibitors were examined. Of all the inhibitors tested in *L. cuprina*, allosamidin was by far the most active compound (Table 2) and was therefore used in further investigations with chitinases from other sources. In the series of less effective inhibitors, compound **II** was of particular interest, since this epoxy-di-*N*-acetylchitotrioside[17] displayed a nearly full inhibition at 1 mM and was suspected to alkylate Asp52 of lysozyme irreversibly by reaction with the oxirane ring system.[44] The glycosyl pyrrolidine and histidine amides were weak inhibitors. Compound **V** could be improved somewhat by extending the number of sugar residues (compound **VI**). Most likely the increased competitive effect in these derivatives was due mostly to the oligosaccharidic unit. The pronounced differences in half-maximal inhibition of chitinases towards allosamidin, ranging from 0·32 μM to 0·69 μM for the arthropods *L. cuprina* and *B. microplus* to 48 nM for *H. contortus* and 1·0 μM for the chitinase Ib from *P. vulgaris*, suggest that there exist significant differences in the catalytic sites of the chitinases themselves. Similar variations for allosamidin sensitivity were observed when chitinolytic enzymes from bacteria, crustaceae and insects were compared, with the *Streptomyces* sp. chitinase being the least (20·9 μM) and *Chironomus tentans* Walk. chitinase being the most (0·46 μM) susceptible enzyme.[40] Since such biochemical screening tools have only recently become available, the search for new chitinase inhibitors with high pesticidal activities has just begun.

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